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Connectivity Mapping (ssCMap) To Predict A20 Inducing Drugs Anti-inflammatory Action In Cystic Fibrosis

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Cystic Fibrosis (CF) lung disease is characterised by a chronic and exaggerated inflammation in the airways. Despite recent developments to therapeutically overcome the underlying functional defect in CFTR (cystic fibrosis transmembrane conductance regulator), there is still an unmet need to also normalise the inflammatory response. The prolonged and heightened inflammatory response in CF is in part mediated by a lack of intrinsic downregulation of the pro-inflammatory NF- κ B pathway. We have previously identified reduced expression of the NF- κ B down-regulator A20 in CF as a key target to normalise the inflammatory response. Here we have used publically available gene array expression data together with sscMap (statistically significant connections' map) to successfully predict drugs already licensed for the use in humans to induce A20 mRNA and protein expression and thereby reduce inflammation. The effect of the predicted drugs on A20 and NF- κ B(p65) expression (mRNA) as well as pro-inflammatory cytokine release (IL-8) in the presence and absence of bacterial LPS was shown in bronchial epithelial cells lines (16HBE14o-, CFBE41o-) and in primary nasal epithelial cells (PNECs) from patients with CF (Phe508del homozygous) and non-CF controls. Additionally, the specificity of the drug action on A20 was confirmed using cell lines with TNFAIP3 (A20) knockdown (siRNA). We also show that the A20 inducing effect of ikarugamycin and quercetin is lower in CF derived airway epithelial cells than in non-CF cells.

A20 (TNFAIP3) | NF-kappaB | Connectivity Mapping | Drug repositioning | CF airway inflammation

Introduction

The response to pathogens, recognised by pattern recognition receptors including Toll-like receptors (TLRs), triggers an acute innate immune response that is mediated by transcription factors such as nuclear factor-kappa-light-chain enhancer of B cells (NF- κ B). NF- κ B activation promotes the transcription of inflammatory mediators in a tightly regulated process. However, in individuals with underlying chronic inflammatory diseases, this regulation is compromised, leading to constitutive NF- κ B activation and persistent inflammation (1-3)

The development of new first-in-class medicines is costly (approximately \$1.2 billion for a single FDA-approved drug) and takes between 10 and 15 years (4, 5). Many newly developed drugs perform well in the preclinical testing, but fail when tested in humans (6). Thus alternative approaches using predictive models to identify new drugs are needed. Gene expression connectivity mapping (www.broadinstitute.org/cmap/) is an advanced bioinformatics technique to establish the connections among biological states via gene expression profiles/signatures. One major application of connectivity mapping is to identify potential small molecules able to inhibit a disease state or regulate the expression of a small number of genes (7-9). We used an advanced version of connectivity mapping, sscMap (statistically significant connections' map) (10), which has been successfully applied to

phenotypic targeting and predicting effective drugs in cancer (10). However, this has not yet been applied to chronic inflammatory diseases.

Cystic Fibrosis (CF) is a chronic multi-organ inflammatory disease, caused by mutations in the CFTR gene (Cystic Fibrosis Transmembrane Conductance regulator) expressed on apical epithelial surfaces. It is the most common lethal genetic disease in Caucasian populations. Lung disease is the primary cause of morbidity and mortality in CF, resulting from dehydration of epithelial surfaces and reduced mucociliary clearance as a consequence of the ionic imbalance created by CFTR mutation. This leads to a cycle of infection and inflammation associated with a progressive reduction in lung function and eventual respiratory failure. A common feature of CF is the heightened, chronic inflammatory response to *Pseudomonas aeruginosa* (*P. aeruginosa*), driven by constitutive NF- κ B activation in airway and peripheral blood cells (2, 3, 11). Primary nasal epithelial cells (PNECs) from patients with the common F508del/F508del mutation and a milder genotype (R117H/F508del), show a significant increase in NF- κ B(p65) which correlates with disease severity (12).

A20 (TNFAIP3) is a central negative regulator of NF- κ B activation following stimulation of TLRs and/or TNF-receptor and regulates different signalling pathways such as NF- κ B and interferon regulatory factor (IRF) signalling (13). A20 modifies

Significance

This study reports that publically available gene array expression data together with statistically significant connections' map (sscMap) can successfully predict already licensed drugs to modify genes of interest. We applied this bioinformatics approach to the NF- κ B regulator A20 (TNFAIP3), which is reduced in Cystic Fibrosis (CF) airway cells. sscMap predicted drugs that should induce A20 and normalise the inflammatory response in CF. Using airway epithelial cells we show that ikarugamycin and quercetin have anti-inflammatory effects mediated by induction of A20. Using siRNA we confirm that the anti-inflammatory effect of ikarugamycin and quercetin are mainly due to A20 induction. We have identified a process whereby already licensed drugs can be successfully repositioned for chronic inflammatory airway diseases.

Reserved for Publication Footnotes

Table 1.

ID of data set	Sample Type	Platform used	Sample size	Published in
GSE2395	Primary nasal epithelial cells (PNECs)	HG-U133A & HG-U133B: GPL96/GLP97	40 (18 CF {F508del homozygous}, 22 controls), exposed to <i>P. aeruginosa</i>	Wright JM <i>et al.</i> 2006
GSE30439	CFBE41o-	HG-U133 Plus2: GPL570	12 (CFBE41o- and CFBE41o- complemented with wildtype CFTR), +/- exposure to <i>PA01</i>	Hampton TH <i>et al.</i> 2012
GSE620	IB3-1	HG-U133A: GPL96	5 (IB3-1 cells, excluded those with 4-phenylbutyrate treatment)	Wright JM <i>et al.</i> 2004
GSE923	Calu-3	HG-U133A: GPL96	19 (Calu-3 {F508del/F508del} infected with <i>P. aeruginosa</i> (muroid vs. motile strain))	Cobb LM <i>et al. J Immunol</i> 2004

Table 2.

Gene symbol	Unique ID	Name	Function
TNFAIP3	202643.s.at	A20	Ubiquitination, negative regulator of NF- κ B
ATF3	202672.s.at	Activating Transcription Factor 3	Binds the cAMP response element (CRE), transcriptional repressor (promoters with ATF sites)
RAB5C	201140.s.at	RAB5C	small GTPase, regulates membrane traffic from plasma membrane to early endosomes, ubiquitously expressed
DENND4A	214787.at	DENN/MADD domain containing 4A	C-myc promoter-binding protein, promotes exchange of GDP to GTP, converting inactive GDP-bound Rab proteins into their active GTP-bound form

classical immune cells (14, 15) as well as epithelial cells (12), endothelial cells (16), embryonic fibroblasts (17), osteoclasts (18) and pancreatic beta-cells (19) and diverse roles for A20 in innate immunity, apoptosis, autophagy and antigen processing (13, 15, 16, 20). Within the innate inflammatory immune response A20 regulates NF- κ B signalling at the level of TRAF6 in mouse embryonic fibroblasts (MEFs) and osteoclasts (17, 18). In cultured human airway epithelial cells, A20 is rapidly induced by viral or bacterial compounds (21) and is essential for termination of the TLR4 signal (22). PNECs stimulated with *P. aeruginosa* LPS show a transient increase in A20, but CF PNECs display lower A20 expression basally and after LPS stimulation (12, 23).

Therefore, A20 induction should have anti-inflammatory effects within the tightly regulated NF- κ B signalling pathway as shown by the induction of A20 through gibberellin (GA₃) in airway epithelial cells. GA₃ induced A20, reduced IL-8 secretion, stabilised cytosolic I κ B α and reduced NF- κ B (p65) activation (24). Here we set out to identify additional compounds able to induce A20. Thus, we performed a compound search using gene expression connectivity mapping to identify existing drugs that could induce A20 expression.

Results

1. Connectivity mapping (sscMap):

The selection of gene array data and creation of the gene signature

Data sets that passed the selection criteria contained human primary nasal epithelial cells (PNECs) and the human bronchial epithelial cell lines CFBE41o-, Calu-3 and IB3-1 analysed basally and after exposure to *P. aeruginosa* LPS (Table 1). In total 76 samples from 4 different published gene array data sets were used. Linear expression correlation and GO enrichment analysis for NF- κ B pathway genes identified the closest correlates to A20. Table 2 shows the top 7 genes that subsequently served as the input to the connectivity mapping process.

Prediction of drugs to induce A20 in airway epithelial cells

This study sought small molecular compounds that may enhance A20 expression and as a confirming negative control, those compounds that may inhibit A20 expression. Table 3 summarizes the top candidate drugs identified. The column entitled 'significance' shows the significance of drugs based on p values and the column 'z-score' shows the correlation of the drugs with the input gene signature. Positive z-scores indicate a positive correlation i.e. the input genes are induced when treated with the particular drug. The significant drugs with the highest positive z-scores along with a negative control were selected for laboratory validation. In addition to p values and z scores, stability of the connections was measured by altering the gene signature and the significance of the connections are given under the column 'perturbation stability'. Drugs with perturbation stability 1 represent strong connections which remain significant with 'perturbation' gene signatures. From these predictions two A20

Figure 1:

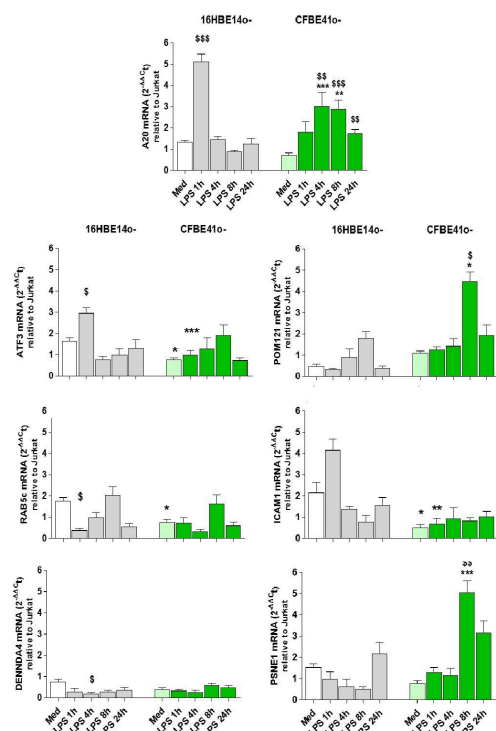


Fig. 1. Gene expression profile of the gene signature genes associated with A20. 16HBE14o- (grey) and CFBE41o- (green) were stimulated (LPS, 10 µg/ml 0-24 h) and mRNA levels of A20, ATF3, Rab5c, DENNDA4, POM121, ICAM-1 and PSNE1 determined as described. \$ significant difference compared to medium control, * significant differences between genotypes.

inducing drugs (ikarugamycin and quercetin) as well as one non-A20 inducing drug (fluvastatin) were chosen for further investigation.

2. Gene expression of gene signature

Expression of the genes identified as the A20/NF-κB gene signature in CF epithelial cells were analysed by qRT-PCR in 16HBE14o- and CFBE41o- cultured in the presence or absence of LPS for 0-24h (Figure 1).

Basal expression: CFBE41o- show significantly lower mRNA expression for A20, ATF3, Rab5c and ICAM1 compared to 16HBE14o- (all $p < 0.05$, $n = 5$). Expression of DENNDA4 and PSNE1 was also lower in CFBE41o- but this did not reach significance.

LPS induced expression: In 16HBE14o-, A20 mRNA is rapidly induced with expression peaking 1h after LPS exposure ($p < 0.001$ compared to medium, $n = 5$), while CFBE41o- show significantly lower (at 1h $p < 0.001$ vs. 16HBE14o-, $n = 5$) and delayed (maximal induction at 4-8 h, $p < 0.01$ and 0.001 vs. medium, $n = 5$) induction upon LPS stimulation. After LPS, ATF3 and ICAM1 expression was significantly lower in CFBE41o- compared to 16HBE14o-

Figure 3:

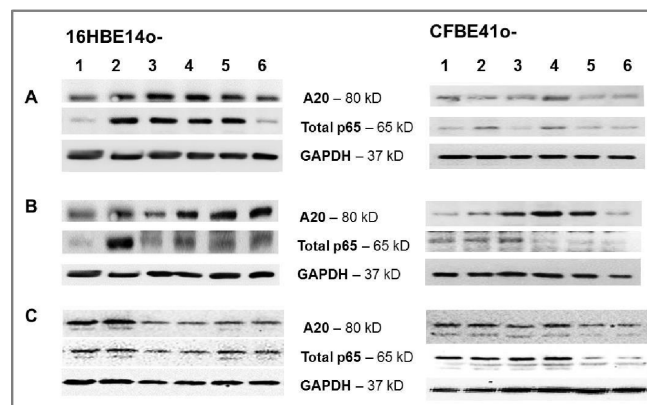


Fig. 2. A20 and p65 mRNA expression of ikarugamycin, quercetin and fluvastatin treated cells. 16HBE14o- and CFBE41o- were pre-incubated with (a) ikarugamycin (0.01, 1 µM), (b) quercetin (0.1, 100 µM) or (c) fluvastatin (0.1, 1 µM) stimulated (LPS, 10 µg/ml, 0-24 h), A20 mRNA determined (qRT-PCR) and expressed as A20/β-actin relative to the internal control.

($p < 0.01$, $n = 5$). Pom121 and PSNE1 expression increased in CFBE41o- compared to medium (8h, $p < 0.05$ and $p < 0.01$) and in CFBE41o- compared to 16HBE14o- (8h, $p < 0.05$ and $p < 0.001$). 16HBE14o- showed a significant reduction in DENNDA4 and Rab5c expression compared to medium (1h, $p < 0.05$ and 4h, $p < 0.05$, respectively), but there was no significant change in the expression of these genes in CFBE41o-.

3. Effect of A20 inducing drugs on cell lines

LDH release in drug exposed 16HBE14o- and CFBE41o-: LDH release was measured after exposure to the drugs alone (0.01–1000 µM) and with LPS stimulation (supplement Figure 1S). Quercetin did not cause any LDH release. Overall fluvastatin was almost without effect on LDH; the exceptions were a slight but statistically significant increase at 10 µM alone (CFBE41o-) and in the presence of LPS (in CFBE41o- and 16HBE14o-). Ikarugamycin (1, 100 µM) caused a significantly higher LDH release in both cell types. In LPS stimulated cells, 0.1 µM ikarugamycin and higher concentrations showed a higher LDH release compared to LPS alone, but this did not reach statistical significance (supplement Figure 1S).

Figure 2:

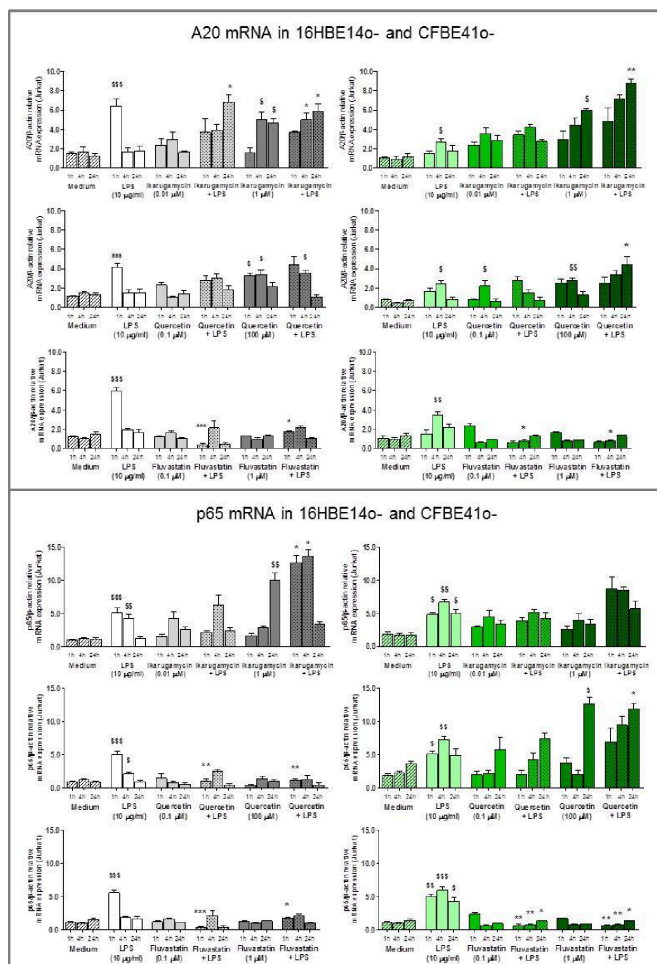


Fig. 3. Effect of ikarugamycin, quercetin and fluvastatin on A20 and p65 protein expression. 16HBE14o- and CFBE41o- were pre-incubated with (a) ikarugamycin, (0.01, 1 µM), (b) quercetin (0.1, 100 µM) or (c) fluvastatin (0.1, 1 µM) and stimulated (LPS, 10 µg/ml, 0-24 h). Cytosolic A20 and p65 protein was determined by Western Blotting: 1 = Ctr, 2 = LPS, 3 = drug at lower conc., 4 = drug at lower conc. + LPS, 5 = drug at higher conc., 6 = drug at higher conc. + LPS.

LPS stimulated IL-8 release in drug pre-treated 16HBE14o- and CFBE41o-: To assess the anti-inflammatory potential of the selected drugs, cells were pre-treated with the drug for 1h, stimulated with LPS and the IL-8 release measured and the relative IC₅₀ calculated (supplement S3). In 16HBE14o-, all drugs reduced IL-8 release by at least 50% with an IC₅₀ of 15.6 µM for ikarugamycin, 0.09 µM for quercetin and 0.11 µM for fluvastatin. In CFBE41o-, only quercetin (IC₅₀ 0.03 µM) and fluvastatin (IC₅₀ 0.001 µM) pre-treatment were able to reduce release by 50%. In contrast, pre-treatment of CFBE41o- with 1 µM ikarugamycin caused a significant increase in IL-8 release compared to LPS alone (LPS 269.9 ± 47.9 pg/ml vs. 590.7 ± 82.6 pg/ml, p<0.05, n=5). Therefore, a meaningful calculation of the relative IC₅₀ for IL-8 release in ikarugamycin treated CFBE41o- cells was not possible.

A20 mRNA induction in drug treated 16HBE14o- and CFBE41o-: To elucidate if ikarugamycin and quercetin facilitate their anti-inflammatory action though the induction of A20 as predicted,

Figure 4:

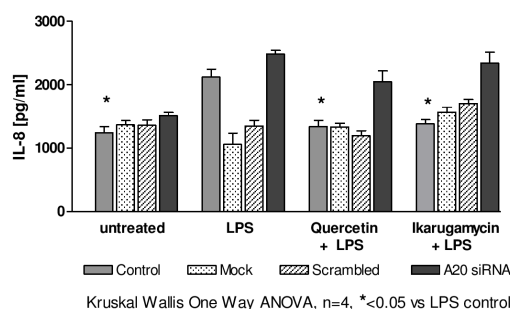


Fig. 4. The anti-inflammatory effect of ikarugamycin and quercetin is mediated by A20 induction. 16HBE14o- with and without knock-down of A20 were pre-incubated with ikarugamycin, (1 µM) or quercetin (100 µM), stimulated and IL-8 analysed. Drug treatment caused a significant reduction in IL-8 (p<0.05) compared to LPS stimulation alone. A20 knock-down resulted in a lack of the anti-inflammatory effect of the drug tested.

A20 mRNA was determined by qRT-PCR. Fluvastatin was included as a negative control. Using the LDH and IL-8 release data two drug concentrations were selected for further investigations (supplement S3). In 16HBE14o-, LPS stimulation caused a significant induction of A20 1h after stimulation (Figure 2). Ikarugamycin (0.01 µM) alone did not cause a significant induction of A20, but additional LPS stimulation caused a significant A20 induction at 24h (p<0.05 vs LPS 24h). 1 µM ikarugamycin significantly induced A20 at 4h on its own, but the higher A20 expression in the presence of LPS (1-24h) did not reach statistical significance. Quercetin pre-treatment did not induce significant levels of A20 mRNA at 0.1 µM, alone or in the presence of LPS. However, at 100 µM quercetin A20 mRNA was significantly induced alone at 1h and 4h (p<0.05 vs. medium 1h and 4h) and in the presence of LPS at 4h (p<0.05 vs. LPS 4h). Fluvastatin alone did not induce A20 mRNA at any time or concentration (Figure 2), in the presence of LPS fluvastatin pre-treatment caused a significant reduction in A20 mRNA at both concentrations tested (p<0.001 for 0.1 µM+LPS 1h vs. LPS 1h; p<0.05 for 1 µM+LPS

Figure 5a:

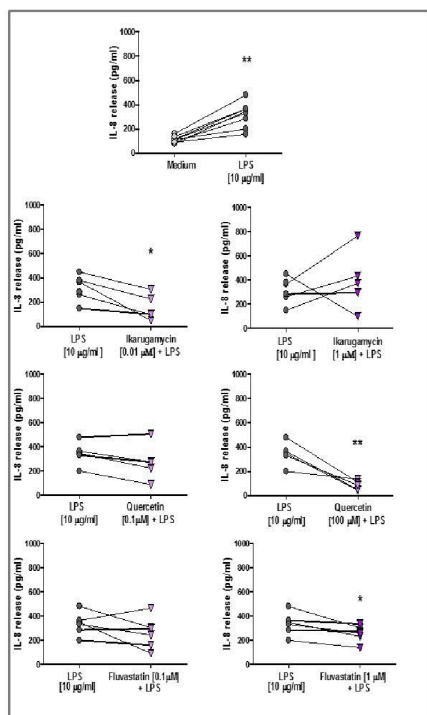


Figure 5b:

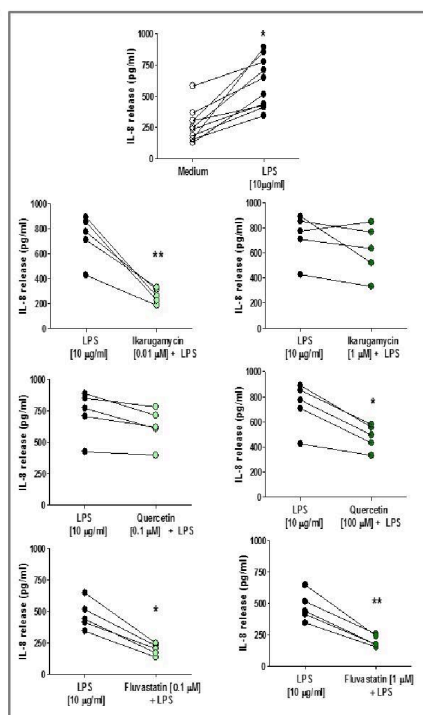


Fig. 5. Effect of ikarugamycin, quercetin and fluvastatin on IL-8 release from PNECs from (a) healthy controls and (b) patients with CF. The release of IL-8 (pg/ml) was determined using a commercially available IL-8 ELISA kit. Statistical analysis was performed using Wilcoxon paired ranked t-test.

1h vs. LPS 1h). In CFBE41o-, LPS significantly induced A20 at 4h ($p < 0.05$), but induction levels were lower than in 16HBE14o- (Figure 2). Ikarugamycin (1 µM) induced significant levels of A20 at 4h and 24h (both $p < 0.05$), this was further increased in the presence of LPS at 4h ($p < 0.05$ vs LPS 4h) and 24h ($p < 0.01$ vs LPS 24h). Quercetin treatment alone significantly induced A20 at 4h at both concentrations ($p < 0.05$ 0.1 µM vs. medium 4h and $p < 0.01$ 100 µM vs. medium 4h). In the presence of LPS, only 100 µM quercetin caused a significant induction of A20 at 24h ($p < 0.05$). Fluvastatin alone did not induce A20 mRNA at any time or concentration (Figure 2), and in the presence of LPS fluvastatin caused a significant reduction in A20 mRNA at both

concentrations tested ($p < 0.05$ for 0.1 µM+LPS 4h vs. LPS 4h; $p < 0.05$ for 1 µM+LPS 4h vs. LPS 4h).

Effect of selected components on NF-κB (p65) mRNA in 16HBE14o- and CFBE41o-: Next we investigated if A20 induction altered NF-κB(p65) mRNA levels (Figure 2). LPS stimulation caused a significant induction of p65 1h after stimulation in 16HBE14o- ($p < 0.01$ – $p < 0.001$ vs. medium). Ikarugamycin (0.01 µM) was without significant effect on p65 mRNA. At 1 µM, ikarugamycin alone induced p65 at 24h ($p < 0.01$ vs. medium 24h) and in the presence of LPS at 1h and 4h (both $p < 0.05$ vs. LPS). Quercetin alone showed no effect on p65 mRNA levels, but when stimulated with LPS both concentrations of quercetin (0.1, 100

Figure 6:

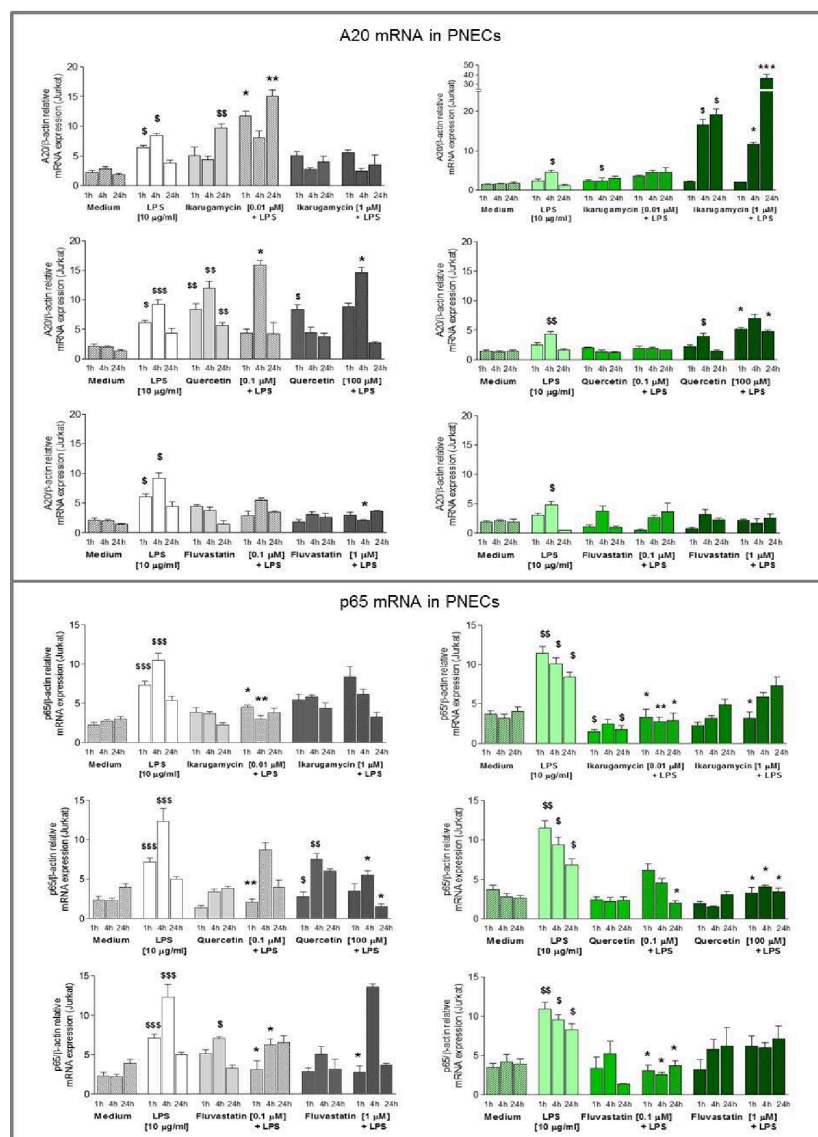


Fig. 6. Effect of ikarugamycin, quercetin and fluvastatin on A20 and p65 mRNA expression in PNECs from (a) healthy controls and (b) patients with CF. Cells were pre-incubated with ikarugamycin, quercetin or fluvastatin at the indicated concentrations (0.01 – 100 μ M) and then stimulated (LPS, 10 μ g/ml, 0–24 h). A20 mRNA was determined by qRT-PCR and expressed as A20/ β -actin relative to internal control. **Table 1: Selected GEO gene expression data** sets selected for the connectivity mapping process. **Table 2: Gene expression profile of NF- κ B/A20 related genes in CF airway disease.** Genes behaving in a similar way than the target gene A20 were determined using linear correlation analyses of the selected gene expression data sets.

Table 3.

Drug	C-score	P-value	Z-score	Significance	Perturbation stability	Drug class
Azacyclonol	0.3496	1.00E-05	4.8417	1	1	γ -Pipradol, ataractive drug
Ikarugamycin	0.4115	6.00E-05	4.1275	1	1	Macrolide type
Quercetin	0.33712	5.00E-05	3.8963	1	1	Flavenoid
Karakoline	0.43145	1.00E-05	3.8033	1	1	Alkaloid Diterpenoid
Fluvastatin	-0.35325	1.00E-04	-3.8267	1	1	HMG-CoA reductase inhibitor

μ M) significantly reduced p65 mRNA levels at 1h ($p < 0.01$ vs LPS 1h). Similar to quercetin, fluvastatin alone showed no effect

on p65 mRNA levels, but after LPS stimulation fluvastatin (0.1, 100 μ M) significantly reduced p65 mRNA levels at 1h ($p<0.001$ and $p<0.05$ vs LPS 1h). In CFBE41o- LPS significantly induced p65 at 1h, 4h and 24h ($p<0.05$ -0.001 vs medium) and overall CFBE41o- exhibited higher expression levels of p65 at 4h and 24h than 16HBE14o- (Figure 2). Ikarugamycin was without significant effect on p65 mRNA expression at any concentration or time point, although overall expression levels appear higher at 1 μ M, when stimulated with LPS (Figure 2). In CFBE41o-, quercetin (0.1 μ M) did not affect p65 mRNA levels. At 100 μ M, quercetin significantly induced p65 ($p<0.05$: quercetin alone 100 μ M 24h vs. medium 24h and quercetin 100 μ M + LPS 24h vs. LPS 24h). Fluvastatin caused a significant reduction in p65 mRNA at both concentrations and all time points after LPS stimulation ($p<0.05$ and $p<0.01$ for 0.1 μ M+LPS vs. LPS; $p<0.05$ and $p<0.01$ for 1 μ M+LPS vs. LPS) (Figure 2).

Effect of predicted drugs on A20 and p65 protein expression:

We then determined the effect of the drugs on cytosolic A20 and p65 protein by Western Blotting using the same selected concentrations than before. Ikarugamycin (0.01 μ M) induced A20 protein in both 16HBE14o- and CFBE41o-, with less A20 protein induction at 1 μ M. Ikarugamycin also induced cytosolic p65 in both cell types (Figure 3a). Quercetin treatment caused a strong induction of A20 protein at both concentrations (0.1, 100 μ M) in 16HBE14o- and to a lower degree in CFBE41o-. Quercetin (100 μ M) reduced cytosolic p65 in 16HBE14o- and in CFBE41o- (Figure 3b). Fluvastatin did not induce A20 protein at either concentration (0.1, 1 μ M) in both 16HBE14o- and CFBE41o- cells. Fluvastatin pretreatment reduced cytosolic p65 protein in 16HBE14o- cells though this was only apparent at the higher concentration in CFBE41o- (Figure 3c).

Specificity of the drug effect on A20 mRNA expression using A20 siRNA: To confirm that the effect of the selected drugs is facilitated through A20 induction, we used siRNA to knock down A20 expression in 16HBE14o- cells as previously described (30). Cells were pre-treated with quercetin or ikarugamycin prior to LPS and IL-8 determined. Results (Figure 4) showed that in 16HBE14o- LPS significantly induced IL-8 ($p<0.05$ compared to untreated control), but when A20 is knocked down IL-8 increased further (although not significantly different from LPS alone). When cells are pre-treated with quercetin (100 μ M) or ikarugamycin (1 μ M), the LPS induced IL-8 release was significantly reduced ($p<0.05$). However, when A20 is knocked down IL-8 levels were not different from LPS control (Figure 4).

Effect of A20 inducing drugs on PNECs Effect on IL-8 release:

LPS significantly induced IL-8 release from PNECs from non-CF and CF patients (non-CF: $p<0.01$, CF: $p<0.05$, Wilcoxon paired test, Figure 5). IL-8 release from CF PNECs was significantly higher than from non-CF PNECs (600.6 \pm 62.8 pg/ml vs. 315.8 \pm 36.1 pg/ml, $p<0.01$, Mann-Whitney test). In non-CF PNECs (Figure 5a), pre-treatment with ikarugamycin at 0.01 μ M, but not at 1 μ M significantly reduced LPS induced IL-8 release ($p<0.05$). In quercetin and fluvastatin treated non-CF PNECs only the higher concentrations tested (quercetin: 100 μ M and fluvastatin: 1 μ M significantly reduced IL-8 release ($p<0.01$ and $p<0.05$, respectively, Figure 5a). PNECs from patients with CF showed similar results with a significant IL-8 reduction at the lower concentration of ikarugamycin (0.01 μ M, $p<0.01$) and the higher concentration of quercetin (100 μ M, $p<0.05$). In CF PNECs fluvastatin treatment significantly reduced IL-8 release at both concentrations tested (0.1 μ M, $p<0.05$; 1 μ M, $p<0.01$, Figure 5b).

A20 induction in PNECs: In PNECs from non-CF control subjects (Figure 6) LPS stimulation resulted in a rapid and significant upregulation of A20 mRNA within 1h and a peak expression at 4h ($p<0.05$, LPS vs. medium at 1h and 4h). Ikarugamycin alone at 0.01 μ M increased A20 mRNA which reached significance at

24h ($p<0.01$) and in the presence of LPS this increase was significantly higher at 1h and 24h than LPS alone ($p<0.05$ and $p<0.01$ vs. LPS). Ikarugamycin (1 μ M) had no effect on A20 mRNA induction, either alone or in the presence of LPS. Quercetin (0.1 μ M) caused a significant induction of A20 compared to medium control at 1h, 4h and 24h (all $p<0.01$) with expression levels similar to those induced by LPS (Figure 6) and this was maintained the presence of LPS, with a peak A20 induction at 4h. The higher concentration of quercetin (100 μ M) significantly induced A20 mRNA at 1h ($p<0.05$) and subsequent stimulation with LPS resulted in significantly increased A20 mRNA levels at 4h ($p<0.05$ vs LPS 4h). Fluvastatin treatment did not induce A20 mRNA expression, but 1 μ M reduced A20 mRNA at 4h LPS (Figure 6).

In CF PNECs LPS induced A20 mRNA expression was lower than in non-CF PNECs, but in CF PNECs LPS induced significant levels of A20 mRNA 4h after LPS ($p<0.05$ vs. medium 4h). Ikarugamycin at 0.01 μ M (alone and in the presence of LPS) had no effect on A20 mRNA levels (Figure 6) but 1 μ M ikarugamycin significantly induced A20 mRNA at 4 and 24h alone ($p<0.05$ vs medium control) and in the presence of LPS ($p<0.05$ and $p<0.001$ vs LPS). Similarly, quercetin treatment with the lower concentration (0.01 μ M) alone and in the presence of LPS had no effect on A20 mRNA levels (Figure 6), while 100 μ M quercetin significantly induced A20 mRNA alone (4h, $p<0.05$ vs medium control) and additionally above LPS induction (1h and 24h, both $p<0.05$ vs LPS). Similar to non-CF PNECs, fluvastatin treatment of CF PNECs had no significant effect on A20 mRNA expression levels.

NF- κ B (p65) induction in PNECs: PNECs from non-CF control subjects respond to LPS exposure with a significant increase in NF- κ B (p65) at 1h and 4h ($p<0.001$ vs medium control). Thereafter, p65 mRNA expression returns to its corresponding medium control value (Figure 6). PNECs from patients with CF however show the expected high levels of p65 throughout the 24h studied ($p<0.01$ at 1h, $p<0.05$ at 4h and 24h vs medium control) (Figure 6).

In non-CF PNECs, ikarugamycin treatment alone did not change p65 mRNA levels (vs medium control), but in the presence of LPS p65 mRNA was significantly reduced ($p<0.05$ at 1h, $p<0.01$ at 4h vs LPS). Overall the higher concentration of ikarugamycin induced p65 mRNA levels with a significant increase at 4h ($p<0.05$ vs medium control). However, in the presence of LPS, p65 levels remained not significantly different from those after LPS exposure at 1h and 24h, but were significantly lower compared to LPS alone at 4h ($p<0.05$) (Figure 6). The lower concentration of 0.01 μ M quercetin alone did not modify basal p65 mRNA. After subsequent LPS challenge p65 mRNA significantly decreased at 1h ($p<0.01$ vs LPS), but then increased in a similar manner to LPS alone. However, 100 μ M quercetin alone significantly reduced p65 induction at 1h and 4h ($p<0.05$ and $p0.01$ vs. medium control). In the presence of LPS this reduction of p65 mRNA reached statistical significance at 4h and 24h (both $p<0.05$ vs LPS) (Figure 6). Fluvastatin (0.1 μ M) induced p65 at 4h ($p<0.05$ vs. medium 4h), but p65 levels remain significantly lower when LPS is added ($p<0.05$, vs LPS at 1h and 4h). The higher concentration of fluvastatin (1 μ M) did not change p65 levels alone, but after addition of LPS, p65 mRNA was initially reduced (1h $p<0.05$ vs LPS) but then induced similarly to LPS alone (Figure 6).

When PNECs from patients with CF were pre-treated with ikarugamycin, p65 levels dropped significantly at 0.01 μ M ikarugamycin in the absence or presence of LPS ($p<0.05$ -0.01). At the higher concentration of 1 μ M p65 levels appeared lower, but this only reached statistical significance at 1h ($p<0.05$ vs LPS) (Figure 6). Pre-treatment of CF PNECs with quercetin did not affect p65 levels alone at either concentration tested, but at

0.1 μM quercetin in the presence of LPS significantly reduced p65 at 24h ($p < 0.05$ vs LPS). Treatment with 100 μM quercetin significantly reduced p65 mRNA in the presence of LPS at all time points (all $p < 0.05$ vs LPS) (Figure 6). Fluvastatin alone had no significant effect on p65 mRNA levels, but significantly reduced LPS induced p65 at 1h, 4h and 24h ($p < 0.05$ vs. medium 1h, 4h or 24h), while the higher concentration of fluvastatin (1 μM) showed no significant effect on basal or LPS induced p65 mRNA (Figure 6).

Discussion

Airways infection and the subsequent inflammation are deleterious for patients suffering from CF. Current drugs targeting the mutated CFTR (potentiators/correctors) improve expression and function of CFTR on epithelial surfaces and patients showed improved lung function and reduced frequency of pulmonary exacerbations, hospitalization and use of intravenous antibiotics, but augmented CFTR function failed to reduce inflammatory markers in sputum (e.g. IL-1, -6, -8) (25) and heterogeneous responses to the treatment have been reported (26), suggesting that CFTR correction/potential may not directly improve the underlying compromised immune response. The negative NF- κB regulator A20 (*TNFAIP3*) is reduced in CF airway epithelial cells, basally and after LPS stimulation (23) and is associated with markers of inflammation and decreased lung function (12). A20 silencing increased TRAF6 and NF- κB activity (18), and A20 over-expression had protective effects in airway inflammation in 'asthmatic mice' (27), suggesting that A20 augmentation normalises the inflammatory response in the airways.

In order to find agents to induce A20 in CF we employed sscMAP, which has been widely used in drug development uncovering potential new indications for existing drugs as well as predicting side effects (28). Using disease specific publicly available gene array data (GEO data sets), we used connectivity mapping to firstly identify the target gene (A20) related gene signature and to secondly predict already licensed drugs to induce A20 expression. We included a total of 76 gene array data from primary nasal epithelial cells and cell lines commonly used in CF research (Table 1). Gene array databases were first selected in August 2013, but a recent (Jan 2016) search revealed no further significant published gene array data on CF (primary nasal) epithelial cells.

The applied linear regression model (Pearson's correlation coefficient) is an established robust method to identify the correlates of a known gene expression estimating the strength of a linear relationship between two random normally distributed variables (29). The application of the linear regression model with GO (gene ontology) selection revealed a gene signature of 6 genes additionally to the seed gene A20 (Table 2) and we confirmed that the expression of these genes is similarly reduced in CF epithelial cells, basally and after LPS stimulation (Figure 1). The identified A20 correlates were *ATF3*, a transcriptional repressor that binds to cAMP response elements (CRE); *RAB5C*, a small ubiquitously expressed GTPase; *DENND4A*, which encodes the C-Myc Promoter Binding Protein (MBP-1); *POM121*, a nuclear transmembrane protein and essential component of the nuclear pore complex; *ICAM1*, a cell surface glycoprotein typically expressed on endothelial and immune cells, especially during inflammation and *PSEN 1* (Presenilin 1), a catalytic component of γ -secretase and a DREAM binding protein. Further descriptions of these genes and their involvements in inflammation can be found in the online appendix. These genes, as a combined gene signature, were then input into the sscMAP process comparing the gene expression of the gene signature with the gene expression in the reference database (www.broadinstitute.org), which was obtained from systematic microarray gene expression profiling.

SscMAP predicted a short list of drugs that should modify the expression profile of the gene signature genes, including A20.

Those drugs included azacyclonol, ikarugamycin, quercetin and karakoline (Table 3). Azacyclonol is a drug used in psychotic individuals (30). We excluded azacyclonol, as its use requires special permission through relevant government authorities. Interestingly, the anti-histamine terfenadine is metabolised to azacyclonol and terfenadine (31). Karakoline is a highly toxic plant diterpenoid (32) and the pharmacological effects of preparations of Aconitum roots are attributed to diterpenoid alkaloids (33). The anti-inflammatory activity of gibberellin (GA_3), also a plant-derived diterpenoid, is mediated through A20 induction (24). We therefore selected ikarugamycin and quercetin for further studies.

Ikarugamycin is a macrolide antibiotic with cytostatic effects against Gram-positive bacteria. We show that ikarugamycin exhibits anti-inflammatory properties in LPS stimulated airway cells. In 16HBE14o- ikarugamycin showed a dose-dependent reduction of LPS-induced IL-8 release (supplement S4), through induction of A20 and reduction of p65 (Figure 2). 16HBE14o- and CFBE41o- did not show reduced cell viability at concentrations lower than 1 μM , higher concentrations increased LDH release suggesting a cytotoxic effect (supplement S2). CFBE41o- appear more sensitive to ikarugamycin treatment (supplement S4), which made it not possible to calculate a meaningful relative IC_{50} value (supplement S3), although p65 protein expression was not increased (Figure 2). In HL-60 cells, ikarugamycin reduced cell viability and increased DNA fragmentation starting at 0.1 μM (IC_{50} of 0.22 μM), while MCF-7 cells and peripheral blood mononuclear cells showed higher resistance. Furthermore, ikarugamycin treatment of HL-60 cells caused a significant caspase activation, increase in intracellular calcium and p38 MAP kinase activation (34). However, investigating the pro-apoptotic mechanisms in bronchial epithelial cells was beyond the scope of this study. Nonetheless, our ikarugamycin data at near cytotoxic levels add valuable information: Firstly, sscMap correctly predicted that ikarugamycin would induce A20 mRNA but sscMap does not predict the physiological effect of the gene induction. CF cells overall show a limited ability to induce A20, however, our results show that – given the right stimulus – CF cells are indeed able to induce A20 mRNA and the high induction of A20 at near cytotoxic levels may be able to counteract the pro-apoptotic stimulation of ikarugamycin.

Quercetin, a flavonoid, is known for its anti-inflammatory effects. *In vivo* studies have shown antioxidant, anti-inflammatory, anti-tumour and even anti-infectious properties of quercetin, which are promoted through its effects on signalling pathways such as NF- κB (35). In lung epithelial cells, quercetin inhibited IL-1 and TNF- α induced I $\kappa\text{B}\alpha$ degradation and NF- κB activity through modification of the MAPK pathway (AP-1) (36). SscMap correctly predicted that quercetin can induce A20 mRNA, adding a new mechanism for the anti-inflammatory effects of quercetin. It also significantly reduced LPS-induced IL-8 release in both cell types with a relative IC_{50} of 0.15 and 0.04 μM in 16HBE14o- and CFBE41o-, respectively. Quercetin at concentrations up to 1000 μM did not show any cytotoxicity, although in neuronal cell cultures quercetin higher than 100 μM was cytotoxic (37). Within the *in vivo* antioxidant network, quercetin has been described to be oxidised and to yield an ortho-quinone, which, in absence of reducing glutathione, can oxidise protein thiols impairing enzyme activities (38). We have not investigated the antioxidant status of our cell culture, but we took precautions to minimise oxidation when preparing our quercetin dilutions.

To further investigate the A20-dependent mechanism of the anti-inflammatory action of quercetin and ikarugamycin, we used A20 knock down in 16HBE14o-. As previously described for the A20 inducing anti-inflammatory compound gibberellin (24), we were able to confirm that the anti-inflammatory effect of the

1089 predicted drugs was indeed mainly mediated by the induction of
1090 A20.

1091 We also tested *fluvastatin*, which was predicted not to affect
1092 or reduce A20 gene expression (negative z-score). Although
1093 fluvastatin exerted anti-inflammatory effects (IL-8) in both cell
1094 lines, our data show that this was not mediated by the induction
1095 of A20 (mRNA), clearly confirming the sscMap prediction. In
1096 asthma, fluvastatin inhibits eosinophil adhesion to ICAM-1 (39)
1097 and fibroblast proliferation (40). Using similar concentrations we
1098 did not observe any reduced proliferation. Fluvastatin at a con-
1099 centration range similar to those we used, reduced basal and LPS-
1100 induced IL-8 release from LPS stimulated whole blood cells, with
1101 CF cells appearing more sensitive to fluvastatin than control cells
1102 (IC₅₀: 19.1 µM in non-CF cells, 4.6 µM in CF blood cells) (41).
1103 In isolated LPS-stimulated peripheral blood monocytes from
1104 patients with chronic kidney disease fluvastatin had a significant
1105 anti-inflammatory effect (IL-8, IL-6) at a concentration range of
1106 0.0001–1 µM (42). Patients with heart transplants receiving 40 mg
1107 fluvastatin/day for 4 weeks showed a significant reduction in total
1108 cholesterol levels and a maximum blood fluvastatin concentration
1109 of 2.11 and 3.77 µM. These studies suggest that we have covered a
1110 physiologically relevant range of fluvastatin. However, fluvastatin
1111 metabolism may be affected by concomitant therapies, especially
1112 substances competing with cytochrome enzymes and in such cases
1113 fluvastatin levels may need to be monitored (43). Any reactions
1114 with other therapies (as they would appear in patients with CF)
1115 were not investigated in our manuscript as they would have been
1116 beyond the scope of the study.

1117 Similar to ikarigamycin, fluvastatin has been described to
1118 have pro-apoptotic effects e.g. in human lymphoma cells, hu-
1119 man smooth muscle cells and in rat neonatal cardiac myocytes
1120 or rat vascular smooth muscle cells (44, 45), mediated through
1121 activation of caspase-3, reactive oxygen species and activation of
1122 p38 MAPK (44, 55). However, statins, through their inhibition of
1123 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), but not through
1124 induction of A20, may still have therapeutic potential in airway
1125 and systemic inflammation in CF (41).

1126 Overall, our study shows that connectivity mapping (sscMap)
1127 can predict A20 inducing drugs. Pre-treatment of cells with both
1128 ikarugamycin and quercetin reduces LPS-induced IL-8 secretion
1129 by induction of A20. In non-CF PNECs both drugs upregulated
1130 A20 and reduced IL-8 and p65 mRNA at lower concentrations
1131 than in cell lines. CF PNECs, however, have a reduced and
1132 delayed A20 inducing response to LPS and to the tested drugs and
1133 a significant A20 induction appears at the higher concentration of
1134 the drugs tested, which might be near to the cytotoxic effect. A20
1135 reduces apoptosis (46, 47) and mutational loss of A20 resulted in
1136 rapid apoptosis and inflammation in hematopoietic cells (48). We
1137 did not determine markers of apoptosis in our study, but the huge
1138 increase in A20 mRNA may indicate a possible counteraction
1139 to pro-apoptotic changes in response to ikarugamycin treatment.
1140 Of particular interest, this may indicate a higher susceptibil-
1141 ity/sensitivity of CF cells to pro-apoptotic stimuli.

1142 Our study has several limitations. Firstly, for the sscMAP
1143 process a huge number of gene array samples are required and
1144 although the database search gave a high number of initial results,
1145 upon detailed inspection several gene array studies could not
1146 be included. Connectivity mapping uses gene array data run on
1147 Affimetrix platforms and we selected those performed on these
1148 platforms. Several published gene array studies were performed
1149 in cell lines. However, the majority of samples selected were
1150 PNECs (n=40), but we also included data from cell lines. Fur-
1151 thermore, every published dataset has been performed using a
1152 specific experimental design with respect to treatments and time
1153 points. We selected experiments that used either no stimulation or
1154 exposure to *P.aeruginosa* LPS or to *P.aeruginosa* itself. A sample
1155 size of 50-100 individual samples is a statistically acceptable sam-

ple size to produce an unbiased result and we used 76 individual
gene array samples.

Secondly, the reference database was generated using differ-
ent cell lines: MCF7, HL60, PC3 and SKMEL5. Although all of
human origin, none of these cell lines are airway derived. We
therefore confirmed the effect of the predicted drugs in airway
relevant and disease specific cell lines, determined an effective
drug concentration in our disease model and confirmed their
effect in primary cells. Additionally, factors such as the interaction
between various signalling pathways and the interplay between
genes can affect the functions of the predicted and validated drugs
when used in humans.

The aim of our study was to investigate the potential of
sscMap to predict A20-inducing drugs from a list of drugs already
licensed for the use in humans to make them available for drug
repositioning. As a proof of concept we have focused on the
LPS induced expression of A20, p65 and cytokines IL-6 and IL-
8. However, in addition to its direct regulation of TLR-induced
NF-κB activation, A20 is also involved in the negative regula-
tion of the of NLRP3 inflammasome via TLR3/4-(TRIF)-RIPK3
(49) may also inhibit inflammation induced regulated necrosis
(necroptosis) via RIPK3 (50), adding further levels of action and
complexity to the anti-inflammatory action of A20. Therefore,
future work analysing further NF-κB driven cytokines such as
TNFα and IL-1β would also indicate if the predicted drugs are
able to modify A20 action on the inflammasome.

Summary

To date there is still a need for alternative anti-inflammatory
drugs for patients with CF as restoring CFTR function with
potentiators and correctors does not directly affect the inherent
innate immune defect. The exaggerated inflammatory response
is in part due to the lack of the NF-κB regulator A20 and
pharmacological induction of A20 is anti-inflammatory. We have
shown here that sscMap is a potent tool to predict effective drugs
that can modify A20 without totally inhibiting NF-κB. This is
particularly important in the clinical setting as pharmacological
suppression of inflammation may increase the incidence of in-
fective exacerbations (51). In addition, A20 inducing drugs have
to be carefully adjusted as in addition to the A20 induction e.g.
ikarugamycin can be pro-apoptotic. A20 inhibits TNF-induced
pro-apoptotic signalling by inhibiting both, the activation of cas-
pase 8 and the activation of c-Jun (52). However, neither the drug
induced pro-apoptotic mechanisms nor the A20 induced anti-
apoptotic mechanisms have been investigated in this study, but
our observation of cytotoxicity despite high anti-apoptotic A20
mRNA levels may suggest an overriding mechanism. Therefore,
while sscMap successfully predicts drugs to modify A20, the effect
of the candidate drugs must to be confirmed in a suitable model
system to optimise treatments.

Our study also suggests that pharmacological induction of
A20 may be less efficient in CF airway cells, but given the appro-
priate stimulation, A20 induction is indeed possible. Tiruppathi *et al.*
recently showed that A20 induction may be regulated not only
via NF-κB, but also through the opposing effects of the repressor
DREAM and transcription factor USF1 (53). Future work will
investigate if modifying the balance between A20 repressor and
transcription factor in CF can more efficiently re-establish A20
induction.

Materials and Methods

Selection of Gene Array data

A search of PubMed GEO data sets (<http://www.ncbi.nlm.nih.gov/gds/>)
was performed in August 2013 using the search terms "Cystic Fibrosis",
"epithelial cells", "airways" and "primary cells". Data sets that passed the search
criteria and were compatible to Affymetrix Human Genome U133A Array
were selected.

Connectivity Mapping (sscMap)

Gene expression profiles were generated using Affimetrix Gene chip
Microarray and the relative expression of treatment vs. control was sorted

1225 in descending order giving rise to ~22000 rank ordered genes and their
1226 expression.

1227 Determination of the gene signature

$$1228 \quad r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

1230 The correlation coefficient r demonstrates the association between A20 and
1231 other genes, either in a positive or negative direction. The significance of
1232 the observed correlations was measured by calculating the corresponding p
1233 values and applying a stringent p value threshold $1/N$ where N is the number
1234 of genes analysed. The significant r values were selected as correlates of
1235 A20. Gene ontology (GO) enrichment analysis (<http://geneontology.org/>)
1236 was then applied to further filter the A20 correlates identifying those
1237 related to NF- κ B. The subsequent gene signature (including A20) was used
1238 as the input query to evaluate the connection between them and the
1239 reference profiles (GEO, Accession Number GSE5258) (8). Based on the
1240 principles of Lamb's connectivity mapping, we used a simpler and more
1241 robust method (9, 10), called sscMap (statistically significant connections'
1242 map), to determine the connections between the gene signature and the
1243 reference profile. The similarity between the gene signature and each
1244 gene expression reference profile was assessed via a connection score.
1245 Connection scores are a function of expression profile and the query gene
1246 signature, which is expected to reflect the underlying connection between

$$1246 \quad \text{Connection strength} = C(R, s) = \sum_{i=1}^n R(g_i) s(g_i),$$

1247 and:

$$1248 \quad \text{Connection Score} = \frac{\text{Connection Strength}}{\text{Maximum possible connection strength}}$$

1249 them.
1250 The sscMap applies a robust and improved scoring system based on the
1251 following formulae:

1252 (Where g_i represents the i th gene in the signature, $s(g_i)$ is its signed rank
1253 in the signature, and $R(g_i)$ is this gene's signed rank in the reference profile).
1254 To calculate the p value, after calculating the connection strength between
1255 a gene signature and the reference profile a large number of random
1256 gene signatures are created and the same number of connection scores
1257 are calculated and the proportion of scores higher than the observed score
1258 in absolute values is the p value. In addition to controlling false positives,
1259 sscMap is extended to measure the stability of the connections discovered by
1260 gene signature perturbation. In order to implement this, one gene is left
1261 out from the gene signature to derive 'perturbation' gene signature and
1262 the changes in the significant connections were observed. The connections
1263 which stay stable over the changes were given the perturbation stability
1264 score, defined as the fraction of times a drug remained significant under
1265 the perturbation process (54).

1266 Cell Culture

1267 The bronchial epithelial cell lines 16HBE140- (control) and CFBE41o- (CF,
1268 F508del/F508del), obtained from D. Gruenert, UCSF, USA were cultured as
1269 described (32). Primary nasal epithelial cells (PNECs) from CF patients (all
1270 F508del/F508del, $n=5$) and healthy volunteers ($n=5$, informed consent given,
1271 research ethics approval 07/NIR02/23) were cultured as previously described
1272 (55). Control participants did not have any acute airways disease at the time
1273 of sampling, or a history of any chronic airways inflammation.

1274 Cell culture stimulations

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1298 Cells were exposed to the selected drugs (ikarugamycin, quercetin,
1299 fluvastatin (all Sigma-Aldrich, SML0188, Q0125, SML0038, 0.01–1000 μ M) at
1300 0–1000 μ M for 1 h prior to LPS stimulation (*P. aeruginosa* LPS, Sigma-Aldrich,
1301 L9143, 10 μ g/mL, up to 24h). Stock solutions of the drugs were kept at -20°C
1302 for up to 3 months. To minimise oxidation or degradation of the compounds,
1303 the working dilutions were freshly prepared.

1304 Determination of LPS induced cytokine release (IL-6 and IL-8)

1305 IL-6 and IL-8 in cell-free culture supernatants were measured by a com-
1306 mercially available ELISA (PeproTech EC Ltd.) according to the manufacturer's
1307 instructions.

1308 LDH Cytotoxicity assay

1309 Lactate dehydrogenase (LDH) release into cell culture supernatants was
1310 determined using a LDH-Cytotoxicity Assay Kit (BioVision Ltd.) according to
1311 the manufacturer's instructions.

1312 Real time qPCR

1313 Total RNA was extracted (GenElute™, RTN350, Sigma-Aldrich) and
1314 quantified. Equal amounts of RNA (250 ng) were reverse transcribed into
1315 cDNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™)
1316 and quantitative RT-PCR performed (LightCycler thermal cycler system,
1317 Roche). Expression of A20, p65 and β -actin were assessed using primer se-
1318 quences previously described (23) and given in supplement table S1. Relative
1319 expression to β -actin was calculated as $\Delta\Delta Ct$. Jurkat cell cDNA acted as an
1320 internal calibrator for all experiments and was used to determine differences
1321 in basal gene expression.

1322 Western Blotting

1323 Cytosolic protein expression was determined by Western Blotting after
1324 extraction in RIPA buffer containing protease inhibitors (cComplete™, Mini,
1325 Roche). Lysates were separated by SDS-PAGE and PVDF membranes incu-
1326 bated with 1 μ g/ml primary antibody: A20 (ab74037, Abcam); p65 (C-20,
1327 Santa Cruz Biotechnology), washed, incubated with appropriate horseradish
1328 peroxidase-conjugated antibody and visualized on a BioRadChemi Doc XRS
1329 system (BioRad). Anti-GAPDH-HRP (ab-9484, Abcam) was used as a loading
1330 control.

1331 Transfections

1332 16HBE140- were seeded at 4×10^4 cells/well and allowed to attach
1333 overnight. Custom FlexiTube siRNA (QIAGEN, UK) was designed against
1334 TNFAIP3 and both cell lines transfected with 50 nM siRNA and Lipofectamine
1335 Transfection Reagent (Invitrogen, UK) over 72h. All experiments included
1336 mock transfection and scrambled controls. Gene silencing was assessed by
1337 qPCR as described above with knockdown of $74\% \pm 7.2$; $n=5$) achieved.

1338 Statistical analysis

1339 All data are presented as the means \pm SEM. Differences between groups
1340 were analysed using the Kruskal-Wallis non-parametric ANOVA with Dunn's
1341 post-test (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). \$ denotes a significant differ-
1342 ence compared to medium control, while * denotes significant differences
1343 between groups (CF vs. non-CF, comparing the same time points or LPS vs.
1344 treatment + LPS). The logarithmic inhibitor concentration versus the relative
1345 IL-8 response achieving a 50% inhibition was calculated as the relative IC_{50} .
1346 GraphPad Prism (La Jolla, California) was used to plot graphs and to analyse
1347 the data.

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